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The objective of this work has been the characterization of chemical markers that are useful for identification of *B. anthracis* using pyrolysis-based methods. The strategy is to select groups of organisms that differ in particular chemotaxonomic features, identify any pyrolysis products that differentiates groups from one another, hypothesize the origin of these chemical markers in the cell, and correlate information from pyrolysis of appropriate model compounds as well as information from independent analytical methods.

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R. S. Sahota, S. L. Morgan, and K. E. Creek, "A Pyrolysis-Gas chromatographic/Mass spectrometric method for measuring DNA content of cultured mammalian cells", *Journal of Analytical & Applied Pyrolysis* **1992**, *19*, 237-49.

R. S. Sahota and S. L. Morgan, "Recognition of chemicals markers in chromatographic data by an individual feature reliability approach to classification", *Anal. Chem.* **1992**, *64*, 2383-2392.

S. L. Morgan, A. Fox, J. C. Rogers, and B. E. Watt, "Identification of chemical markers for microbial differentiation and detection by gas chromatography-mass spectrometry", invited chapter in: *Modern Techniques for Rapid Microbiological Analysis*, W. Nelson, Ed., VCH Publ. Inc, NY, 1992.

R. S. Sahota and S. L. Morgan, "Vector representation, feature selection, and fingerprinting: An application of pattern recognition to pyrolysis-gas chromatography/mass spectrometry of nucleosides", *Anal. Chem.* **1993**, *65*, 70-77.

E. L. Nimz and S. L. Morgan, "On-line derivatization for complex fatty acid mixtures by capillary gas chromatography/mass spectrometry", *J. Chromatogr. Sci.*, **31**, 145-149 (1993).

M. K. Higgins, R. S. Bly, S. L. Morgan, and A. Fox, "Differentiation of isomeric alditol hexaacetates and identification of aldohexoses by electron impact mass spectrometry", *Anal. Chem.* **1994**, *66*, 2656-2668.

S. L. Morgan, B. E. Watt, and R. C. Galipo, "Characterization of microorganisms by pyrolysis GC, pyrolysis GC/MS, and pyrolysis MS", in: *Applied Pyrolysis Handbook*, T. Wampler, Ed., Plenum Press, NY, 1995.

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Rachhpal S. Sahota, Ph.D., University of South Carolina, May 1991. "Computer pattern recognition applied to pyrolysis-gas chromatography/mass spectrometry for the detection of cancer markers".

Bruce E. Watt, Ph.D., University of South Carolina, May 1991. "Identification and Characterization of Chemical Markers Produced from Microorganisms Including *Bacillus Anthracis* by Pyrolysis-Gas Chromatography/Mass Spectrometry."

Erik L. Nimz, Ph.D., University of South Carolina, August 1993, "Capillary Gas Chromatography/mass Spectrometry following Analytical Transformation of Molecules of Biological Significance".

Melinda K. Denny, Ph.D., University of South Carolina, August 1994, "Chemometric Analysis of Gas Chromatographic/Mass Spectrometric Data for Classification and Modeling of Chemical Structure and Composition".

BRIEF OUTLINE OF RESEARCH FINDINGS

Background

The objective of this work has been the characterization of chemical markers that are useful for identification of *B. anthracis* using pyrolysis-based methods. The strategy is to select groups of organisms that differ in particular chemotaxonomic features, identify any pyrolysis products that differentiates groups from one another, hypothesize the origin of these chemical markers in the cell, and correlate information from pyrolysis of appropriate model compounds as well as information from independent analytical methods.

Systematic differences between different bacterial species, genera, and families have been long defined in chemical terms. Closely related species can often be differentiated by the presence or prominence of characteristic compounds (chemical markers). Microorganisms have a noncompartmentalized cytoplasm in which there are few organized structures other than the chromosome. There are only a limited number of potential chemical markers present in the cytoplasm. The bacterial cell envelope (cell membrane, surrounding cell wall, and outer membrane if present) offers a rich assortment of highly unusual polymers that have distinct monomeric constituents. These markers include amino acids, carbohydrates, fatty acids, and quinones. Optimal use of these markers for differentiation or detection requires understanding the significance of the presence of specific chemical components in bacterial macromolecules and in what different taxonomic categories they are present (1,2).

Chemical markers are often present as monomeric components of a polymer. Either the markers must be released intact or a recognizable related compound must be generated. The marker or derived compound must be volatile, thermally stable, and sufficiently inert for GC/MS. With derivatization methods, depolymerization is achieved by acid hydrolysis, methanolysis, or saponification. Active functional groups on a monomeric compound are derivatized (acylated, esterified, *etc.*) to inert forms suitable for GC/MS. In pyrolysis methods, depolymerization of bacterial components is done by rapid heating, either by capacitive discharge across a filament or by Curie-point heating, and the thermal products are rapidly swept into the

GC/MS for analysis. Pyrolysis GC/MS generates a chemical profile of an organism that is derived from the carbohydrate, fatty acid, lipid and other cellular components (2,3).

It is vital in these studies that the organisms be well characterized prior to testing. For certain organisms, such as *Bacillus anthracis* and *Legionella pneumophila*, taxonomic identification schemes are not completely defined. To clarify the relatedness of bacterial species or to validate the presence of specific chemical components within organisms, carbohydrate or fatty acid profiling by high resolution capillary GC-MS represents a complementary chemotaxonomic tool. Naturally, the most useful chemical markers are those that are completely unique to a particular group of microorganisms; such markers do exist and can be detected by analytical pyrolysis. More commonly, if the chemical marker is not unique, it must be proven that differences substantial enough to allow discrimination exist. Often multivariate information provided by a combination of several unique and/or nonunique, but discriminating, markers can completely determine a taxonomic decision. Basing analytical pyrolysis GC-MS methods on invariant structures also provides reproducibility between different instruments and laboratories. The recognition of bacteria in the presence of potentially interfering biological matrices and reliability of decisions based on this information is of primary concern. That unique pyrolysis products can be generated from microorganisms was demonstrated by identification of dianhydroglucitol, derived by dehydration from glucitol phosphate residues in the group-specific polysaccharide of group B streptococci (4-6). Ideally, pyrolysis products are preferred that retain as much of the structural integrity and chemical uniqueness of the original monomer, rather than small molecules that could be derived from multiple sources (2).

Identification of chemical markers for aldohexoses

As carbohydrates constitute a larger class of microbial constituents, we have focused on analysis of carbohydrates by pyrolysis and by derivatization GC/MS. Figure 1 shows a total ion pyrogram for a sample of pure glucose. The final pyrolysis temperature setting was 650 ° in a CDS Pyroprobe, and capillary chromatography was performed on 30 m DB-1701 bonded-phase fused-silica. Selected peaks are identified in Table 1. Many early eluting pyrolysis products are small fragments common to the pyrolysis of all the aldohexoses and of little use in identifying which aldohexose is present in a sample. The anhydrosugars, 1,6-anhydro-glucopyranose (peak 12) and 1,6-anhydro-glucofuranose (peak 13), are the most useful pyrolysis products for the identification of glucose. Another anhydrosugar characteristic of glucose is 1,4:3,6-dianhydro-α-D-glucopyranose (peak 9). The structures of these chemical markers are shown along with their corresponding mass spectra in Figure 2. Some of the pathways for formation of these prominent pyrolysis products from carbohydrates are shown in Figure 3. Anhydrosugars are formed by transglycosidation. Polycondensation of the monosaccharides at elevated temperatures forms random polymers having linkages involving the C-1 glycosidic carbon. Polymer bonds are broken by pyrolysis through intraglycosidation forming the stable 1,6-anhydrohexoses. Figure 3A shows structures of several prominent anhydrosugar pyrolysis products from glucose. A large peak of 5-hydroxymethyl-2-furaldehyde (peak 11) is indicative of the presence of any hexose in a sample. In addition to being present in pyrograms of aldohexoses, 5-hydroxymethyl-2-furaldehyde is also formed by pyrolysis of ketoses. The observation that the uncondensed portion of the monosaccharide is the likely source of the dehydation products furaldehyde (peak 5) and 5-hydroxymethyl-2-furaldehyde (peak 11) has been previously made by Helleur *et al.* (7) based on the work of Ohnishi *et al.* (8). Figure 3B shows structures for the proposed degradation mechanism that produces these markers. The intermediate compound, 3-deoxyglycosulose, has been identified by Kato and Komorita (9) in support of this mechanism.

Figure 1. Pyrogram of glucose. See Table 1 for identification of labeled peaks.

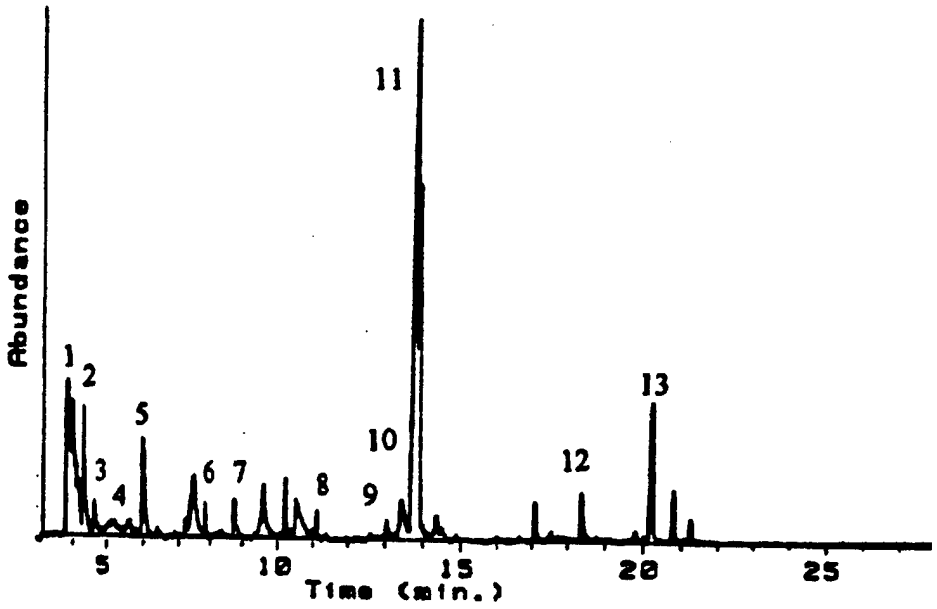


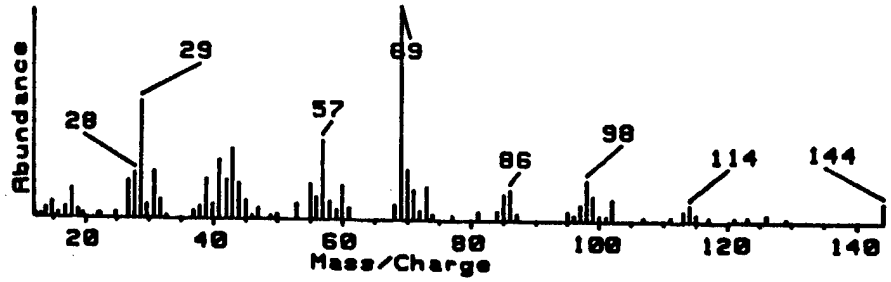
Table 1. Pyrolysis products identified in the pyrogram of glucose. Peak numbers are labeled in Figure 1.

Peak Number	Compound	Retention Time, min	Identification
1	carbon dioxide	3.89	m, l
2	1,2-dihydroxyethene	4.31	l
3	hydroxypropanone	4.68	m, l
4	(2H)-furan-3-one	5.63	l
5	2-furaldehyde	6.02	m, l
6	5-methyl-2-furaldehyde	7.85	m, l
7	1,3-dihydroxy-2-propanone	8.80	m
8	2,3-dihydro-3,5-dihydroxy-(4H)-pyran-4-one	11.12	m
9	1,4:3,6-dianhydro-α-D-glucopyranose	13.07	l
10	2-hydroxymethyl-2,3-dihydropyran-4-one	13.29	l
11	5-hydroxymethyl-2-furaldehyde	13.80	m, l
12	1,6-anhydroglucopyranose	18.44	l
13	1,6-anhydroglucofuranose	20.18	l

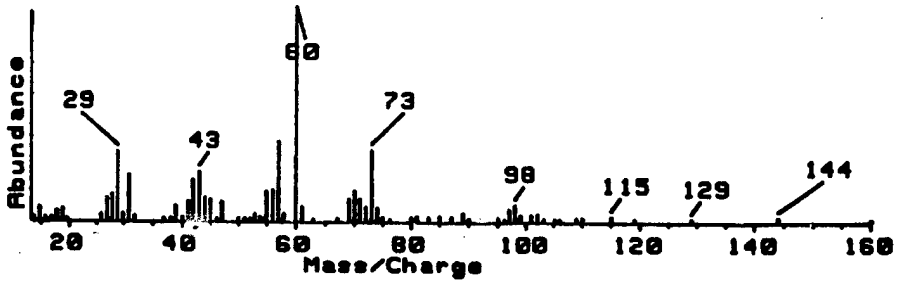
Identification by: m, EI mass spectrum library search; l, literature reference

Figure 2. Structures and mass spectra of three anhydrosugar chemical markers produced by pyrolysis of glucose.

(A) 1,4:3,6-dianhydro-α-D-glucopyranose, retention time 13.1 min.



(B) 1,6-anhydroglucopyranose, retention time 18.4 min.



(C) 1,6-anhydroglucofuranose, retention time 20.2 min.

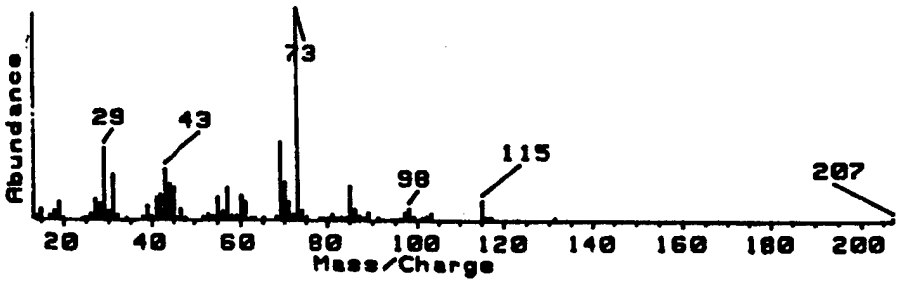
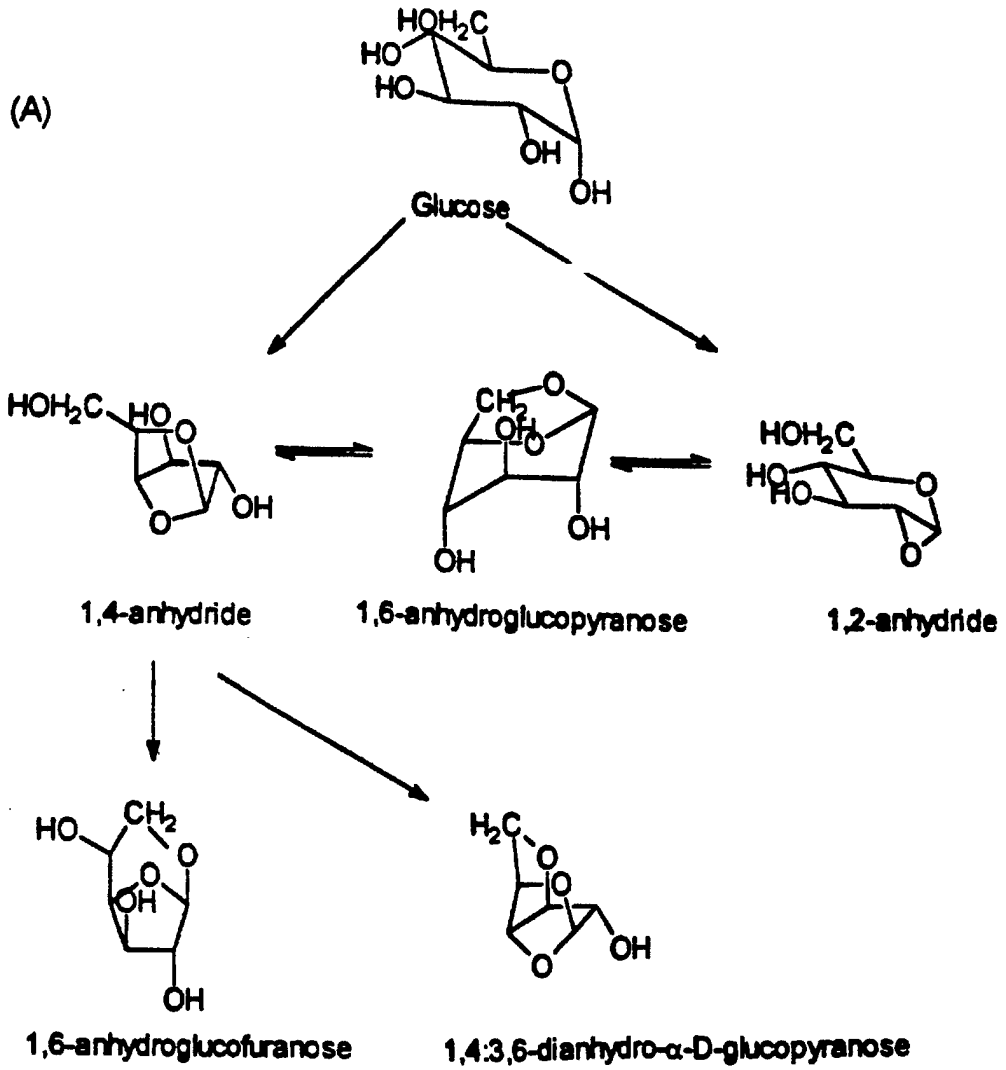
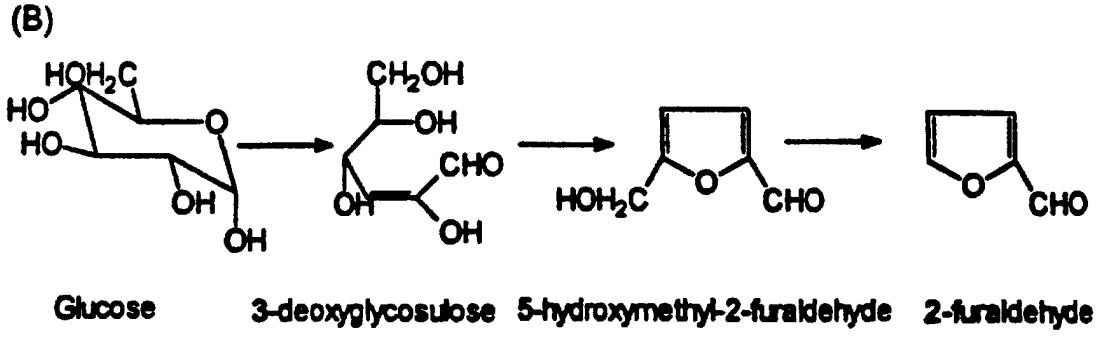


Figure 3. Simplified pathways for formation of prominent pyrolysis products from glucose.

(A) anhydrosugar chemical markers.



(B) furans.



Pyrolysis results are similar for other aldohexoses (Table 2). Peak identifications are based on literature references, mass spectral library searches, and comparison with authentic samples. Because the anhydrosugar products are isomers, they have very similar mass spectra. High resolution capillary GC provides sufficient differentiation by retention time for unique identification of the peaks. This procedure was also used to assign identities to other pyrolysis products, such as the dianhydrohexopyranoses. The dianhydrohexopyranose marker was only observed in some of the aldohexoses. It is absent in galactose, talose, idose, and gulose due to stereochemical blocking of the formation of the dianhydro structure by the hydroxyl group on C-4.

Table 2. Anhydrosugar pyrolysis products characteristic of aldohexoses.

Compound	Retention time, min	Target mass ion, m/z[a]
Glucose		
1,4:3,6-dianhydro-α-D-glucopyranose	13.07	69 29 57 43 41
1,6-anhydroglucopyranose	18.44	60 57 73 29 43
1,6-anhydroglucofuranose	20.18	73 69 29 43 31
Galactose		
1,6-anhydrogalactopyranose	16.78	60 73 57 29 43
1,6-anhydrogalactofuranose	18.91	73 69 43 29 31
Mannose		
1,4:3,6-dianhydro-α-D-mannopyranose	12.24	69 29 57 41 31
1,6-anhydromannopyranose	17.61	60 57 29 73 43
1,6-anhydromannofuranose	17.88	73 43 29 69 44
Talose		
1,6-anhydrotalopyranose	18.15	60 73 57 29 43
1,6-anhydrotalofuranose	19.14	73 71 60 29 43
Idose		
1,6-anhydroidopyranose	17.61	60 42 43 57 73
1,6-anhydroidofuranose	18.62	73 43 31 44 57
Allose		
1,6-anhydroallopyranose	16.47	60 73 57 29 43
1,6-anhydroallofuranose	17.82	73 43 29 31 44
Altrose		
1,6-anhydroaltropyranose	18.13	60 57 73 29 44
1,6-anhydroaltrofuranose	19.09	73 44 29 43 69
Gulose		
1,6-anhydrogulopyranose	16.87	60 31 42 57 43
1,6-anhydrogulofuranose	17.83	73 31 42 42 60

[a]Five most abundant masses listed in decreasing order of abundance.

Despite their chemical and mass spectral similarity, the combination of retention time and mass spectra together enable recognition and assignment of the chemical identity of anhydrosugar pyrolysis products. These experiments show that anhydrosugars of each aldohexose retain sufficient stereochemistry to be useful as markers for the parent monosaccharide. Although previous work has reported the existence of these anhydrosugars as products of monomeric carbohydrates, this work demonstrates the generality and reliability of anhydrosugar chemical markers for monomeric carbohydrates. These results also indicate the possibility of rapid identification and analysis of individual aldohexoses present within larger carbohydrate containing compounds found in microorganisms and other biologically-derived samples.

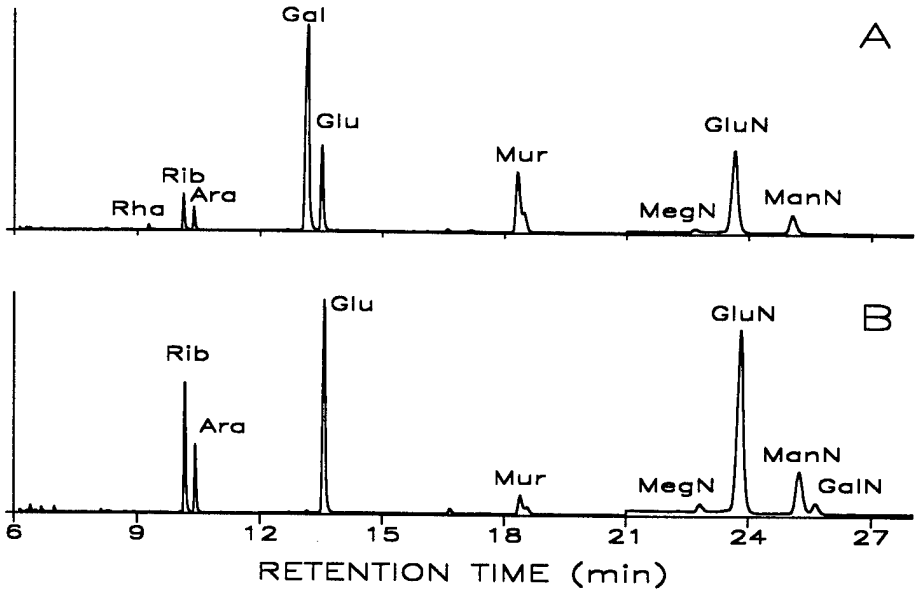
Analysis of carbohydrate monomers in microbial polysaccharides by pyrolysis and GC/MS

Poly-β-hydroxybutyrate (PHB), a lipid substance, is a polymer of 3-hydroxybutyric acid, and serves as a major energy and carbon storage compound in bacteria. Diverse bacteria usually accumulate PHB in intracellular granules having an approximate composition of 98% PHB and 2% proteins and small amounts of lipids. Although several pyrolysis studies have characterized butenoic acids produced by PHB in microorganisms, the use of trans-2-butenoic acid as a chemical marker for PHB in diverse organisms had not been previously validated. In a recent study we have validated a pyrolysis-based analytical method for the determination of PHB content by comparison with an extraction derivatization method (10). Using selected ion monitoring GC/MS, PHB was detected in five of eight microorganisms. The amount of PHB as a percentage of the dry weight of the whole cell was highest in two Legionella and two Bacillus strains and only low amounts of PHB were detected in the one Streptococcus strains. No PHB was detected in the other three organisms studied (*P. vulgaris*, *E. coli*, and *S. aureus*).

Pyrolysis GC/MS of a pure PHB standard produces 3-butenoic acid, cis-2-butenoic, and the major product trans-2-butenoic acid. The mechanism for this process has been suggested to occur by hydrogen transfer through a six-membered ring rearrangement reaction. The area of the ion at mass m/z 86 in the largest peak (trans-2-butenoic acid) was selected for quantitative measurement of PHB in microorganisms. Higher levels of 2-butenoic acid were present in the pyrograms of the two Legionella strains and the two Bacillus strains. All other microorganisms generate less 2-butenoic acid on pyrolysis, with the levels shown for *P. vulgaris*, *E. coli*, *S. pyogenes*, and *S. aureus* indistinguishable from background. Reconstructed ion pyrograms of mass m/z 86 were used to show the presence of PHB in B. *anthracis*. The derivatization and pyrolysis results on the same organisms correlate well with one another ($r^2 = 0.902$), although absolute magnitudes are not directly comparable between pyrolysis and derivatization. The actual amount of a pyrolysis product depends on accessibility of a component to release from its intracellular matrix; derivatization GC-MS may give a more accurate picture of the microbial composition. Two recent studies complement our results nicely. DeLuca, *et al.* (11) have demonstrated the correlation of results from Curie-point pyrolysis tandem MS with fatty acid content in bacteria. Snyder, *et al.* (12) have also characterized underivatized lipids from microorganisms using Curie-point pyrolysis coupled to short-column capillary GC and ion trap MS.

Demonstration of production of components of the lethal toxin of *B. anthracis* is widely accepted as the most reliable way of identifying anthrax strains. Such approaches can not be used to characterize many of the avirulent strains we plan to use in the present study since they lack the genes coding for these toxins (13). *B. anthracis* can be differentiated from *B. cereus* and *B. thuringensis* with monoclonal antibodies which recognize the cell wall galactose-N-acetylglucosamine polysaccharide (13). The presence or absence of the two virulence plasmids (PXO1 and PXO2) does not affect differentiation of *B. anthracis* from closely related bacilli (13). We are working with avirulent strains provided by Dr. Stephen Leppla of Ft. Detrick, MD. We performed carbohydrate profiling by derivatization GC/MS on six strains of *B. anthracis* and three strains of *B. cereus* (ATCC 6464, 12826 and 14579). As seen in Figure 4, the presence of galactose (Gal) was found to reliably distinguish the two species.

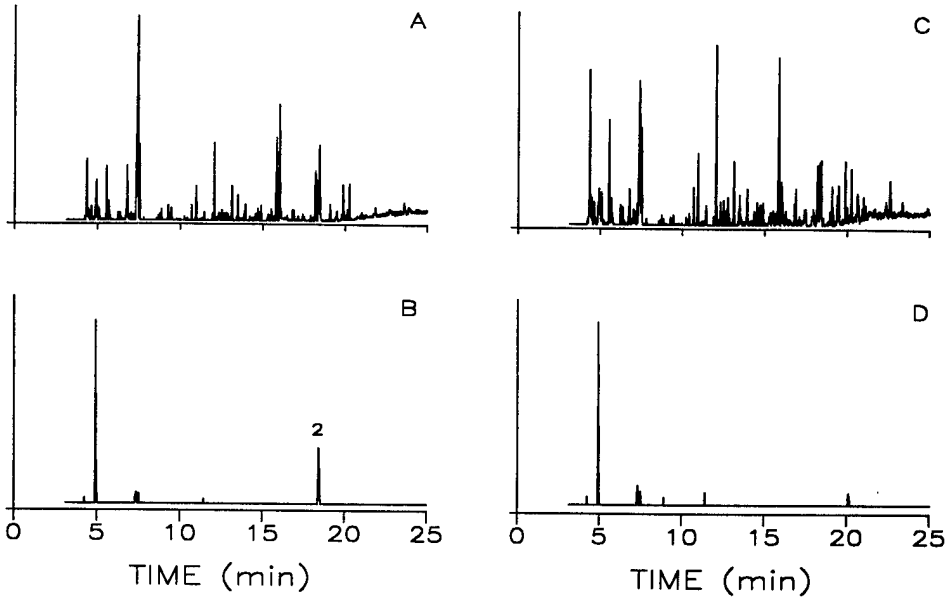
Figure 4. Alditol acetate carbohydrate profiles (GC/MS selected ion chromatograms) of (A) *B. anthracis* (NNR-1), and (B) *B. cereus* (ATCC 6464). Arabinose (Ara) and methylglucamine (MegN) were internal standards for neutral sugars and amino sugars respectively. Abbreviations: rhamnose (Rha), ribose (Rib), galactose (Gal), glucose (Glu), muramic acid (Mur), glucosamine (GluN), mannosamine (ManN), and galactosamine (GalN).



Previous work in our laboratory (14) demonstrated that simple carbohydrates (monomers, dimers, and trimers) could be differentiated from one another by pyrolysis GC. Building on that work, Helleur *et al.* (15-17) have shown that pyrolysis GC/MS can be used to identify stereoisomers of pentoses and hexoses and have applied the method to carbohydrate profiling of oligosaccharides and heteropolysaccharides. Galactose, in particular, was noted to produce 1,6-anhydro-galactopyranose. We have applied this information to differentiation of *B. anthracis* strains. A peak at the matching retention time in the pyrogram of a *B. anthracis* sample (peak 2 in Figure 5) has the identical mass spectrum (not shown) as 1,6-anhydro-galactopyranose from galactose. A comparative pyrogram of a related organism, *B. cereus* (Figure 5C and D), does not show a peak at the same retention time. The area of peak 2 using m/z ion 60 was selected for quantitative measurement of 1,6-anhydro-galactopyranose in these pyrograms. Agreement between two sets of replicate derivatization and pyrolysis analyses for six strains of *B. anthracis* was excellent with a R^2 value of 0.929. Clearly, this anhydrosugar product can differentiate *B. anthracis* strains. More strains/species of bacilli and other organisms need to be characterized to assess the utility of this chemical marker.

We have also studied bacterial lipopolysaccharide (LPS), the unique glycolipids found in Gram negative bacteria. LPS contains a repeating oligosaccharide sequence bonded to a core polysaccharide bound to a lipid complex. Although the basic structure of LPS's are similar, their exact composition can vary and provide chemotaxonomic information for bacterial species and strain identification. Figure 6 shows pyrograms of LPS isolated from different bacterial species. The presence of the 1,6-anhydro-glucopyranose peak at 18.4 min and the 1,6-anhydro-glucofuranose peak at 20.1 min in the pyrograms of the two strains of *E. coli* (Figure 6A and 6B) suggest the presence of glucose. The *E. coli* strain 0127:B8 (Figure 6B) additionally contains galactose, as indicated by the 1,6-anhydro-galactopyranose peak at 16.8 min (peak 4) and the 1,6-anhydrogalactofuranose peak at 18.91 min (peak 5).

Figure 5. Total ion abundance pyrograms of (A) *B. anthracis* (NNR-1) and (C) *B. cereus* (ATCC 6464). Reconstructed ion m/z 60 pyrogram of (B) *B. anthracis* (NNR-1) and (D) *B. cereus* (ATCC 6464); peak 2 is 1,6-anhydro-galactopyranose.



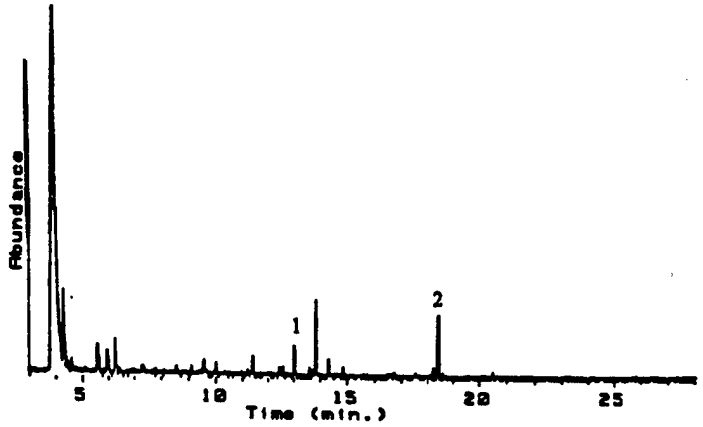
The *K. pneumoniae* LPS is known to contain a complex heptasaccharide consisting of three glucopyranosyl units, and one each of galactopyranosyl, galactofuranosyl, rhamnopyranosyl, and glucouronosyl units (17). The aldohexose content of this LPS is confirmed by Py-GC/MS results shown in Figure 6C. The presence of the 1,6-anhydroglucopyranose peak at 18.4 min and the 1,6-anhydroglucofuranose peak at 20.1 min are indicative of the glucose monomer, while the 1,6-anhydrogalactopyranose at 16.8 min is indicative of galactose. Note also that the relative amounts of these pyrolysis products are different in the three pyrograms of Figure 6, suggesting differing relative amounts in the microbial LPS samples.

Quantitative applications of analytical pyrolysis

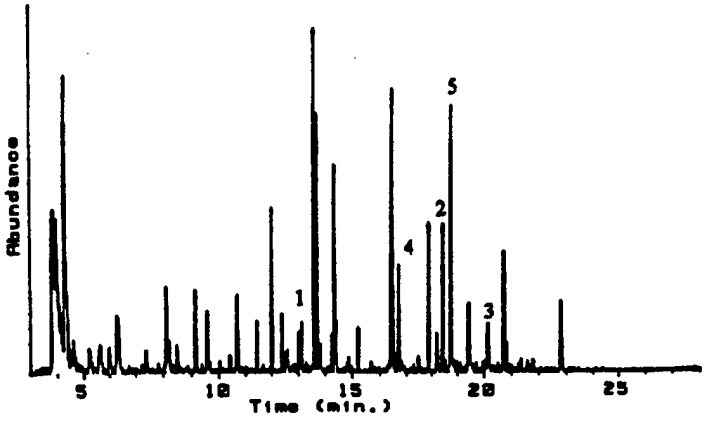
We have had a continuing interest in quantitative applications of pyrolysis. We have already mentioned our study of a chemical marker for poly-β-hydroxybutyrate (PHB), 2-butenoic acid, in microbial pyrograms. The use of 2-butenoic as a chemical marker was validated by correlation with results from an extraction, hydrolysis, and derivatization GC/MS method for determination of PHB, by growth trials profiling PHB content as function of culture age, and by comparison of pyrograms from diverse organisms. In another study (18), we used furfuryl alcohol as a chemical marker to measure DNA content of mammalian cells by Py-GC/MS . The results were compared to results from an established colorimetric method based on reaction with diphenylamine. N-Acetylglucosamine was employed as an internal standard for calibration of the method. The single step procedure for DNA determination involved minimal sample manipulation and had a detection limit of about 100 nanograms of DNA. In both of these cases, quantitation achieved by pyrolysis was validated by comparison to other independent analytical methods.

Figure 6. Total ion pyrogram of the two bacterial lipopolysaccharides. Peak identities: (1). 1,4:3,6-dianhydro-α-D-glucopyranose; (2). 1,6-anhydroglucopyranose; (3) 1,6-anhydroglucofuranose; (4) 1,6-anhydrogalactopyranose; (5) 1,6-anhydrogalactofuranose.

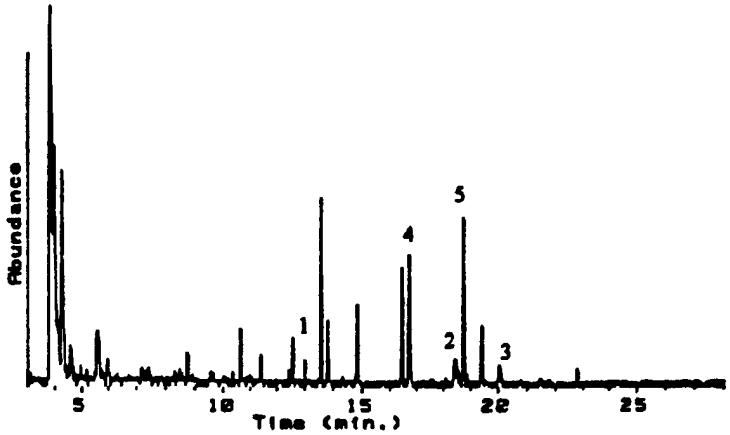
(A) *E. coli.* Strain 026:B6;



(B) *E. coli.* Strain 0127:B8;



(C) *K. pneumoniae.*



Chemometrics and pattern recognition with analytical pyrolysis

Results from our laboratory have focused on two aspects of applying chemometrics to GC/MS data. Sahota and Morgan (19) developed an approach for representing chromatograms as vectors using mass spectra to validate peak comparisons. Feature selection was employed to produce standard vectors that enhance discrimination between different classes of samples. The data representation process was integrated with feature selection to facilitate fingerprinting and recognition of chemical markers. Pyrolysis-gas chromatography/mass spectrometry fingerprints for five nucleosides were generated from replicate analyses. Unknowns were classified by Euclidean and Mahalanobis distance measures from these representative fingerprints. A combination of fingerprinting and feature selection is also shown to be effective in recognizing unique chemical markers for different classes of samples. A second paper from our laboratory (20) applied a discriminant analysis algorithm based on the classification reliability of individual features GC and GC-MS data. Two feature selection methods were investigated in which feature weights were defined either by Fisher ratios or by classification accuracy in single feature dimensions. The discriminant function employed was a linear function of these feature weights where the coefficients of summation were reliability values, ranging from -1 to +1, measuring the confidence with which classification can be made based upon that feature alone. This approach provides information useful for the recognition of unique chemical markers characteristic of particular groups of samples.

Finally, in a recent paper (21), we used a combination of principal component, cluster analysis, and multivariate hypothesis testing on cluster means to statistically validate differentiation of alditol hexaacetates of hexoses by electron impact mass spectrometry. Isomeric aldohexose sugars derivatized using the alditol acetate method produce diastereomeric products that can be differentiated by electron impact (EI)-mass spectrometry. The use of sodium borodeuteride instead of sodium borohydride for reduction in the derivatization scheme enables identification of aldohexoses because their hexitol hexaacetate products retain the asymmetry of the starting sugar. The observed differences in the EI-mass spectra were visualized using principal component analysis and cluster analysis. The statistical significance of these differences was evaluated using Hotelling's T^2 statistic for the comparison of multivariate cluster means. The electron impact mass spectra of nondeuterated and deuterated hexitol hexaacetates permit differentiation of the diastereomeric nondeuterated hexitol hexaacetates as well as identification of all of the aldohexoses.

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